Anaerobic Growth of Microorganisms with Chlorate as an Electron Acceptor

ÅSA MALMQVIST,^{1*} THOMAS WELANDER,² AND LARS GUNNARSSON¹

ANOX AB, Ideon Research Park, S-223 70 Lund,¹ and Department of Applied Microbiology, University of Lund, S-221 00 Lund,² Sweden

Received 18 January 1991/Accepted 12 May 1991

The ability of microorganisms to use chlorate (ClO_3^-) as an electron acceptor for respiration under anaerobic conditions was studied in batch and continuous tests. Complex microbial communities were cultivated anaerobically in defined media containing chlorate, all essential minerals, and acetate as the sole energy and carbon source. It was shown that chlorate was reduced to chloride, while acetate was oxidized to carbon dioxide and water and used as the carbon source for synthesis of new biomass. A biomass yield of 1.9 to 3.8 g of volatile suspended solids per equivalent of available electrons was obtained, showing that anaerobic growth with chlorate as an electron acceptor gives a high energy yield. This indicates that microbial reduction of chlorate to chloride in anaerobic systems is coupled with electron transport phosphorylation.

Chlorate, ClO_3^- , is a highly oxidized compound and should therefore be suitable as an electron acceptor in anaerobic respiration. However, since chlorate, which does not occur naturally, was introduced into the environment only by the activities of humans, time has been short; from an evolutionary point of view, for development of the enzymatic system needed for the utilization of chlorate as an electron acceptor. Nevertheless, enzymatic conversion of chlorate by different types of microorganisms has been demonstrated by a number of investigators (4, 7, 13). Both nitrate reductase and chlorate reductase have been shown to catalyze the conversion of chlorate to chlorite, and nitrate reductase has also been suggested to catalyze the reduction of iodate to iodide (15). In *Proteus mirabilis*, nitrate reductase is formed under anaerobic conditions in the presence of nitrate, while in the absence of nitrate, chlorate reductase is formed instead (13). It has been stated that *P. mirabilis* can use chlorate as a terminal electron acceptor in anaerobic electron transport (7), but microbial growth with chlorate as a terminal electron acceptor in an energy-yielding reaction was not demonstrated.

The potential involvement of microorganisms in chlorate conversion is interesting from an environmental point of view. Removal of chlorate from bleach effluents from the pulp and paper industry by means of biological treatment under anaerobic conditions has been demonstrated by several investigators in Sweden (3, 8, 9, 18). Chlorate is formed and ends up in the effluent when chlorine dioxide is used for pulp bleaching. The elimination of chlorate from the effluent is highly desirable, since chlorate has toxic effects on aquatic plants. It has been shown that bladder wrack (*Fucus vesiculosus*) is sensitive to chlorate in concentrations over 20 $\mu\text{g/liter}$ (14). Although it has been demonstrated that chlorate can be removed by means of biological treatment, the mechanism and the products of chlorate removal have not been determined. Knowledge about the products formed is important, since chlorite, one possible product of chlorate removal, is considered more toxic than chlorate itself.

The aims of this study were to determine whether chlorate can be used by microorganisms as an electron acceptor

under anaerobic conditions and to determine the end products of chlorate removal in anaerobic complex microbial systems.

MATERIALS AND METHODS

Analyses. Chlorate and chlorite were analyzed by means of an ion-exchange chromatography method. The sample (10 to 100 ml, depending on the expected chlorate concentration) was passed through an anion exchanger (Dowex 1 $\times$ 8, 200 to 400 mesh, Cl^- form; Carl Roth KG, Karlsruhe, Germany) in which chlorate and chlorite were retained. The resin was washed with 50 ml of distilled water. Chlorite was eluted with 50 ml of 0.8 M sodium chloride solution, after which chlorate was eluted with 100 ml of 1.6 M sodium chloride solution. The chlorite fraction (25 ml) was mixed with 10 ml of 0.3 M potassium iodide solution, 5 ml of 2 M sulfuric acid, and 10 ml of distilled water, resulting in an oxidation of iodide to iodine. The amount of iodine formed was determined by titration with 0.0125 M sodium thiosulfate solution and with starch as indicator. The chlorate fraction (20 ml) was treated for 2 min with 40 ml of concentrated hydrochloric acid in an evacuated vacuum bottle. A reservoir with a stopcock was connected to the vacuum bottle, allowing the addition of reagents without releasing the vacuum in the bottle. The reservoir was washed with 30 ml of distilled water. A total of 20 ml of 0.3 M potassium iodide solution was then added and followed by another washing with 20 ml of distilled water. The iodine formed was determined by means of iodometric titration as described above.

Chloride was analyzed by potentiometric titration as described previously (2). Acetate was determined by gas chromatography as described previously (10).

The bacterial biomass in the chemostat was determined as the amount of volatile suspended solids (VSS) (2). The quantitation of VSS is based on filtration through a glass-fiber filter (Whatman 934 AH) followed by drying and ignition of the sample. The difference in weight between the dried and ignited samples is used for calculation of the VSS.

Nitrogen, methane, carbon dioxide, and hydrogen sulfide in the gas phase were analyzed by gas chromatography, as described previously (16).

Batch tests. The batch tests were carried out in 155-ml

* Corresponding author.

serum bottles. The bottles were filled with 100 ml of distilled water containing sodium chlorate and acetic acid in different concentrations. The pH of the medium was adjusted to 7 with sodium hydroxide. The amounts of nutrients, e.g., N and P, in the complex inoculum were considered sufficient, and so no extra nutrients were added to the medium. Nitrogen gas was bubbled through the medium in order to obtain a low oxygen level in the bottles. Each bottle was then inoculated with 1 ml of activated sludge from the Sjölanda municipal treatment plant (Malmö, Sweden) and sealed with a butyl rubber stopper. The bottles were then incubated at 37°C for 9 days. The samples were analyzed for chlorate, chlorite, chloride, and acetate before and after incubation.

Continuous test. The continuous test was carried out in a 400-ml double-jacketed glass chemostat. The temperature was maintained at 37°C by means of recirculation of temperature water from a thermostat bath through the outer jacket of the reactor. The pH was kept at 7 by automatic titration with 1 M NaOH. The chemostat was carefully sealed and tested for gas leaks to ensure that no significant amount of oxygen would diffuse into the system, but no measures were taken to remove dissolved oxygen from the medium. Aerobic microorganisms are known to rapidly deplete small amounts of oxygen fed to a complex microbial system, establishing anaerobic conditions, and even if the feed is being saturated with dissolved oxygen, the amount of oxygen being introduced into the chemostat this way would, at the most, give a consumption of about 1% of the acetate fed into the system. A defined medium containing 1 to 15 mM NaClO₃, 16.7 to 25 mM acetic acid, 3 mM NH₃, 0.25 mM KH₂PO₄, 0.1 mM MgSO₄, 0.1 mM Ca(OH)₂, 0.05 mM FeSO₄ · 7H₂O, 0.05 mM MnSO₄ · H₂O, 5 mM NiCl₂ · 6H₂O, 5 mM CoCl₂ · 6H₂O, 5 mM ZnSO₄ · 7H₂O, 0.1 mM H₃BO₃, 0.1 mM Na₂SeO₄, 0.1 mM Na₂WO₄, and 0.1 mM Na₂MoO₄ was used.

The reactor was filled with 360 ml of medium (1 mM sodium chlorate and 16.7 mM acetate) and 40 ml of activated sludge from the Sjölanda municipal treatment plant and run batchwise for 24 h, after which a continuous flow of medium was started, which gave a dilution rate of 0.04 h⁻¹. This dilution rate was maintained throughout the experiment, but the chlorate concentration in the feed was increased stepwise to a final concentration of 15 mM. As the chlorate concentration was increased from 7 to 10 mM, the acetate concentration was increased from 16.7 to 25 mM. At each chlorate level, at least 10 reactor volumes were allowed to pass through the reactor before the concentrations were increased. The chlorate, chloride, and acetate concentrations as well as the VSS were analyzed daily.

RESULTS

Batch tests. Chlorate was consumed and chloride was formed in equimolar amounts in the bottles (Table 1). Chlorite was not detected in any of the samples. Acetate was consumed as chlorate was reduced (Table 1). The chlorate removal was incomplete in bottles in which the original acetate concentration was low compared with the chlorate concentration, while all acetate was consumed in these bottles. On the other hand, all chlorate was reduced while significant amounts of acetate remained in bottles in which the original acetate concentration was high compared with the chlorate concentration.

Continuous test. After each increase in the chlorate concentration in the feed, a complete removal of chlorate was

TABLE 1. Amounts of chlorate, chloride, and acetate before and after incubation in batch tests

Test	Concn (mM) of ^a :						
	ClO ₃ ⁻		Cl ⁻			Ac ⁻	
	Before	After	Before	After	Exp. ^b	Before	After
1	0.92	0.62	0.35	0.72	0.65	0	0
2	0.92	0.40	0.35	0.89	0.88	0.17	0
3	2.00	1.28	0.35	1.05	1.08	0.17	0
4	0.65	0	0.29	0.96	0.94	1.70	0.83
5	1.31	0	0.29	1.66	1.59	1.70	0.17

^a Before and after, before and after incubation in batch tests.

^b Exp., expected chloride concentration, calculated from the theoretical amount of chloride produced in the chlorate reduction plus the contribution from the inoculum.

achieved within four retention times. Chloride was formed and chlorate was removed in equimolar amounts (Table 2). Acetate was consumed and biomass was produced in the chemostat. The acetate consumption and the biomass production increased with an increasing chlorate concentration in the feed (Table 2). The growth yield varied within the range of 1.9 to 3.8 g of VSS per equivalent of available electrons. The gas production in the chemostat was very low and could not be measured properly. Up to 15.8% of the gas phase consisted of carbon dioxide, and the rest was nitrogen gas.

Microscopic investigation of the reactor content revealed a >85% enrichment of motile bacteria with a helical morphology (Fig. 1). No particles other than bacteria (e.g., organic precipitates) that could have contributed to the VSS and given an overestimation of the biomass were observed.

DISCUSSION

The results of the batch and continuous tests clearly show that chlorate can be reduced to chloride in an anaerobic complex microbial system. The reduction of chlorate to chloride in the experiments was accompanied by a consumption of acetate, and since a reduction reaction has to be coupled with an oxidation reaction, acetate must have been oxidized. The detection of carbon dioxide in the gas phase of the chemostat shows that at least part of the consumed acetate was completely oxidized to carbon dioxide and water. Since only the gas composition, and not the gas production, was determined, it is not possible to make a complete carbon balance around the chemostat. However, from the observation that the gas production was very low

TABLE 2. Amounts of chloride and VSS formed and acetate consumed at different influent chlorate concentrations in the continuous test

Chlorate	Concn (mM) of:		Amt (mg/ml) of VSS
	Chloride	Acetate	
1.0	1.08	5.4	58
2.0	2.08	4.8	82
3.0	3.12	5.3	100
4.0	4.03	6.7	120
5.0	4.88	8	136
7.0	6.86	11.5	311
10.0	10.07	15.3	505
15.0	14.98	22.5	640

FIG. 1. Interference photomicrograph of the chlorate enrichment culture. Bar, 10 μm .

compared with the amount of liquid feed, and from theoretical considerations based on the fact that carbon dioxide was the only gas being produced in the chemostat, it can be inferred that the unknown amount of carbon leaving the chemostat with the gas phase must have been small compared with the amounts of carbon leaving with the liquid phase in the forms of carbonic acid and bicarbonate. The amounts of the dissolved species can be estimated from the known content of carbon dioxide in the gas phase and from previously reported data on the carbon dioxide gas-liquid equilibrium constant (6) and the dissociation constants for carbonic acid (17). Thus, for the carbon dioxide content of 15.8% in the gas, obtained at the highest chlorate concentration in the medium (15 mM), it can be calculated that 51% of the carbon in the consumed acetate left the chemostat in the form of carbonic acid and bicarbonate. By assuming an average formulation of biomass of $\text{C}_5\text{H}_9\text{O}_3\text{N}$ (12), 54% of the carbon in the consumed acetate can be accounted for by the 0.64 g of biomass produced per liter. Altogether, this gives a recovery of 105% of the carbon from the consumed acetate, without accounting for the negligible amounts of carbon dioxide in the gas and carbonate. With consideration of the uncertainty of the analyses, the risk that data from other studies on equilibrium constants may not be completely valid for the actual system studied, and the sensitivity of the calculated result to even small deviations from pH 7 in the chemostat, the somewhat more than 100% recovery is well within the margin of error. The result of the carbon balance calculation suggests that the following reaction occurred in the chemostat:

The partial consumption of chlorate in the bottle without any added acetate in the batch test can most likely be explained by the presence of organic compounds in the complex inoculum, which could act as electron donors for the chlorate reduction.

The fact that a considerable amount of biomass was produced in the chemostat strongly suggests that microorganisms which are able to derive energy from the reaction in equation 1 exist. Since no methane was formed in the system, no other energy-yielding reactions, not involving chlorate, can have provided enough energy for a biomass production of the magnitude obtained in the experiment. The maximum contribution of aerobic growth to the biomass

FIG. 2. Measured and calculated acetate consumption at different influent chlorate concentrations in the continuous test.

production can be estimated, assuming that the feed was saturated with dissolved oxygen (7 mg/liter) and that the aerobic growth yield on acetate is 3 g per equivalent of available electrons (11). By this calculation, less than 1% of the biomass produced at the highest concentration of chlorate in the feed could have been caused by growth with oxygen. In the same way, it can be calculated that sulfate reduction could theoretically have produced up to 1% of the biomass. However, no hydrogen sulfide was detected in the gas phase, indicating that no sulfate reduction took place in the chemostat. No nitrate was present in the medium.

It can be seen from equation 1 that each mole of chlorate oxidizes 0.75 mol of acetate. Thus, since all chlorate was reduced to chloride in the chemostat, the amounts of acetate consumed in the catabolic reaction at the different influent chlorate concentrations can be calculated. In addition to being consumed for catabolic purposes, acetate was used as a carbon source in the production of biomass. The acetate consumed in the anabolism can be estimated from the amounts of biomass formed. By using the chemical formulation of biomass given above, it can be calculated that 19 mmol of acetate should be used up as a carbon source per gram of biomass formed. Thus, the total consumption of acetate can be estimated from the following equation:

$$\text{Total acetate consumed (mmol)} = \{[0.75 \times \text{chlorate removed (mmol)}] + [19 \times \text{VSS produced (g)}]\} \quad (2)$$

The total acetate consumption calculated from the chlorate removed and the biomass produced according to equation 2 can be compared with the measured consumption of acetate at the different influent chlorate concentrations (Fig. 2). This comparison shows that the calculated values are in accordance with the measured values, except for those at the lowest concentration of chlorate in the feed. This agreement further verifies that a complete oxidation of acetate to carbon dioxide and water according to equation 1 occurred in the experiments.

The standard free energy change at pH 7 of equation 1 can be calculated to -132 kJ/mol of electrons exchanged from the free energies of formation of the reactants and products, as described previously (5, 17). This can be compared with the free energy changes of respiration with other electron acceptors, such as oxygen, nitrate, and sulfate:

Thus, from a strictly thermodynamic point of view, chlorate should be an even more favorable electron acceptor than oxygen. However, it is evident from earlier investigations (9, 18) that chlorate reduction does not normally take place in aerobic environments, showing that oxygen is preferred to chlorate as an electron acceptor in complex microbial systems. Nevertheless, the growth yield of 1.9 to 3.8 g per equivalent of available electrons obtained in the experiment was of the same magnitude as the aerobic growth yield on acetate of 2.1 to 3.1 g per equivalent of available electrons reported for different pure cultures in other studies (1, 11). This shows that anaerobic growth with chlorate as an electron acceptor gives a high energy yield, suggesting that microbial chlorate reduction to chloride is coupled with electron transport phosphorylation. However, the mechanism of the conversion is largely unknown. The reaction may be entirely enzymatic, or it may involve both enzymatic and purely chemical steps. It also remains to be investigated whether the ability to utilize chlorate as an electron acceptor is common among microorganisms or whether it is limited to certain specialized species.

ACKNOWLEDGMENTS

Financial support from Eka Nobel AB and the Swedish Board of Technical Development is gratefully acknowledged.

REFERENCES

1. Abbott, B. J., and A. Clamen. 1973. The relationship of substrate, growth rate, and maintenance coefficient to single cell protein production. *Biotechnol. Bioeng.* **15**:117-127.
2. American Public Health Association. 1975. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, Washington, D.C.
3. Axegård, P., L. Gunnarsson, and Å. Malmqvist. 1989. Biologisk metod att reducera kloratutsläpp. *Sven. Papperstidn.* **5**:50-52.
4. Azoulay, E., S. Mutaftschiev, and M. L. Martins Rosade de Sousa. 1971. Étude des mutants chlorate-résistants chez *Escherichia coli* K12. III. Mise en évidence et étude de l'activité chlorate-réductase C des mutants *chl* C⁻. *Biochim. Biophys. Acta* **237**:579-590.
5. Brock, T. D., and M. T. Madigan. 1988. Energy calculations, p. 790. In M. T. Madigan and T. D. Brock (ed.), *Biology of microorganisms*, 5th ed. Prentiss-Hall International, Inc., Englewood Cliffs, N.J.
6. Callander, I. J., and J. P. Barford. 1983. Precipitation, chelation, and the availability of metals as nutrients in anaerobic digestion. I. Methodology. *Biotechnol. Bioeng.* **25**:1947-1957.
7. de Groot, G. N., and A. H. Stouthamer. 1969. Regulation of reductase formation in *Proteus mirabilis*. I. Formation of reductases and enzymes of the formic hydrogenlyase complex in the wild type and in chlorate resistant mutants. *Arch. Microbiol.* **66**:220-233.
8. Eckerman, P. 1987. Nu skall blåstången tillbaka till Ödängla. *Nord. Cellulosa* **7**:48-49.
9. Germgård, U., and T. Berglund. 1987. Kloratutsläpp kan undvikas med hjälp av luftade dammar. *Sven. Papperstidn.* **6**:36-39.
10. Hansson, G., and N. Molin. 1981. End product inhibition in methane fermentations: effects of carbon dioxide on fermentation and acetogenic bacteria. *Eur. J. Appl. Microbiol. Biotechnol.* **13**:242-247.
11. Mayberry, W. R., G. J. Prochazka, and W. J. Payne. 1967. Growth yields of bacteria on selected organic compounds. *Appl. Microbiol.* **15**:1332-1338.
12. McCarty, P. L. 1964. Anaerobic waste treatment fundamentals. Part 1. Chemistry and microbiology. *Public Works* **1964**:107-112.
13. Oltmann, L. F., W. N. M. Reijnders, and A. H. Stouthamer. 1976. The correlation between the protein composition of cytoplasmic membranes and the formation of nitrate reductase A, chlorate reductase C and tetrathionate reductase in *Proteus mirabilis* wild type and some chlorate resistant mutants. *Arch. Microbiol.* **111**:37-43.
14. Rosemarin, A., J. Mattsson, K. J. Lehtinen, M. Notini, and E. Nylén. 1986. Effects of pulp mill chlorate (ClO₃⁻) on *Fucus vesiculosus*—a summary of projects. *Ophelia* **4**(Suppl.):219-224.
15. Tsunogai, S., and T. Sase. 1969. Formation of iodide-iodine in the ocean. *Deep-Sea Res.* **16**:489-496.
16. van Huyssteen, J. J. 1967. Gas chromatographic separation of anaerobic digester gases using porous polymer. *Water Res.* **1**:237-242.
17. Weast, R. C., D. R. Lide, M. J. Astle, and W. H. Beyer (ed.). 1989. Handbook of chemistry and physics, 70th ed. CRC Press, Boca Raton, Fla.
18. Yu, P., and T. Welander. 1990. Biological removal of chlorinated organics from kraft bleach effluent, p. 490-495. In *Proceedings of the 24th EUCEPA Conference*, May 8 to 11, 1990. Control, maintenance, environment, new available techniques and current trends. Arbor Publishing, Stockholm.